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JOURNAL OF AGRICULTURAL RESEARCH
DEPARTMENT OF AGRICULTURE

VOL. V WASHINGTON, D. C., OCTOBER 18, 1915 No. 3

ENZYMS OF APPLES AND THEIR RELATION TO THE RIPENING PROCESS

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INTRODUCTION

Several years ago the writer, at that time connected with the Washington State Agricultural Experiment Station, in cooperation with Mr. N. O. Booth, the horticulturist of that Station, undertook an investigation of the possibilities of slowing up the ripening of fruits by means other than cold storage. While these investigations were in progress, Mr. Booth severed his connection with the Station, but it was understood that he would continue the studies in his new location. For that reason no report of our observations at that time has ever been published; but, since no publication of the results of further work along this line has appeared, the writer feels at liberty to assume that the investigation has been discontinued and to discuss briefly the observations which were made, since they form the starting point for the studies to be reported in this article.

Since the term "ripening" is used to designate various different stages in the development of fruit, it is first necessary to define it as it will be used in this article. Seeds upon ripening usually lose water and go into a resting stage from which germination may take place. But the flesh of an apple (*Malus* spp.) or similar fruit has no definite connection with the life history of the embryo of the seed; hence, its "ripeness" can not be measured in terms of the germination ability of the seed. The fruit itself goes through the following stages of development. There is first a period during which the fruit is growing—i. e., increasing its weight of dry matter. At the end of this period, no matter whether the fruit remains on the tree or is picked off, growth ceases and chemical changes set in which result in the development of the characteristic odor and flavor and later in the disintegration of the flesh of the fruit. When this disintegration proceeds far enough, the fruit becomes soft, mushy, or overripe, and usually at either this or some preceding stage organisms of decay gain entrance to the tissues, and the fruit rots. In the absence

of infection with any germs of disease or decay, the fruit loses water and shrivels up to a withered mass. The group of changes that take place during the second of these stages—i. e., the period between the cessation of growth and the disintegration of the tissue until it becomes soft or mushy—will be termed the "ripening process."

The object of all storage or preservation of fresh fruit is to slow up the ripening process and so to prolong this period as much as possible. It is a well-known fact that temperature has an important effect upon the rapidity with which these changes take place. It was the object of the studies referred to above to determine whether other factors also influence the rate of these changes and whether they are due in part to infection with disease germs or are wholly enzymic in character.

Two general methods of study were attempted. First, an attempt was made to surround individual apples with a film or coating which would prevent gaseous exchanges and bacterial infection. Repeated efforts to secure a perfect film of this sort with a variety of different materials proved failures; so this method was abandoned. The second method involved the sealing up of the apples in atmospheres of different pure gases under as nearly sterile conditions as possible in order to prevent both disease infection and the ordinary gaseous exchanges. Several large glass bottles, each capable of holding about a peck of apples, were fitted with tight stoppers provided with a glass inlet tube reaching to the bottom of the bottle and an exit tube extending just through the cork. Carefully washed apples were rinsed in a dilute solution of formaldehyde, followed by distilled water, and immediately introduced into the jars and the stoppers sealed in. The apples were of the Alexander variety and were almost ripe—i. e., they would only keep a few days longer without becoming soft. After sealing in the stopper the inlet tube was connected to a supply of pure gas and the latter passed through until no air could be detected in the gas issuing from the exit tube, when the glass tubes were melted off, thus effectively sealing the jars. This method did not, of course, remove the air contained in the tissues of the apples themselves, but this was relatively small in amount.

Each of five jars was filled with one of the following gases: Hydrogen, nitrogen, oxygen, carbon dioxid, and sulphur dioxid; a sixth was sealed with its ordinary air content. No moisture-absorbing material was placed in the jars, as it was thought that this would produce abnormally rapid losses by evaporation from the tissues of the fruit. Further, the recognized chemical changes in the fruit during the ripening process are probably not influenced by the moisture content of the surrounding air, so that the saturation of the air in the jars with water vapor evaporated from the fruit would not be likely to influence the nature of these changes, while constant absorption of this vapor would mean rapid shriveling of the fruit.

The jars were left in a warm, light laboratory and were examined from time to time. The apples in air continued to ripen normally and

in about four weeks were visibly overripe, the lower ones beginning to collapse under the pressure of the weight of the upper layers. Those in oxygen seemed to ripen a little more rapidly, but the difference was not nearly so great as had been expected and was hardly enough to warrant any conclusion that pure oxygen hastened the ripening process. Those which were surrounded by nitrogen and hydrogen did not soften so noticeably, but became discolored and unhealthy in appearance, a phenomenon later observed and reported by Hill (8).[1] After some 8 or 10 weeks, however, these apples also softened into a mushy mass. The apples in carbon dioxid and in sulphur dioxid remained apparently firm and unchanged for a long time, except that the latter gas completely bleached the skins of the apples in its jar, leaving them a uniform creamy white in color. After nearly six months had elapsed, these jars were opened and the fruit examined. That which had been in an atmosphere of sulphur dioxid was firm and solid, but was, of course, so thoroughly impregnated with the disagreeable gas that its quality could not be judged. The apples which had been in carbon dioxid were firm in flesh, possessed the characteristic apple odor, although the gas in the jar had a slight odor of fermented apple juice, and were not noticeably injured in flavor.

It appeared, therefore, that the phenomena ordinarily associated with ripening were greatly inhibited by an atmosphere of carbon dioxid, but that the cause of this inhibition was not wholly a lack of oxygen. It seemed that the changes taking place in the apple were not simple respiratory changes, but probably in large part were internal enzymic activities.

The experiment was repeated the following summer, using raspberries, blackberries, and loganberries instead of apples. It was found that berries which softened in 3 days in air would remain firm for from 7 to 10 days in an atmosphere of carbon dioxid. At this point the studies were interrupted by a change in professional engagements and have not been resumed.

Recently, Hill (8) reported a series of observations so similar in character that interest in the matter was revived; and opportunity being presented for a systematic study of the enzyms of apples by a graduate student[2] working under the writer's direction, such a study was undertaken, with the results reported below.

CHANGES IN CHEMICAL COMPOSITION OF APPLES DURING RIPENING

The changes in the chemical composition of apples during ripening have been very thoroughly studied by Bigelow, Gore, and Howard (2). The report of their investigations contains a careful review of the literature on the subject, together with significant contributions from the

[1] Reference is made by number to "Literature cited," p. 116.

[2] The writer's thanks are due to Miss Inez Everett, the graduate student who assisted in the preparation of the material for examination and the carrying out of the several tests.

work of the authors themselves. Briefly summarized, the results of these investigations show that the principal changes which take place in the apple during ripening are as follows:

(1) A slight but continuous decrease in total acidity calculated as malic acid.

(2) A gradual decrease in sucrose.

(3) A gradual increase at first, followed by a later slight decrease, in invert sugar and total carbohydrates calculated as invert sugar.

(4) The disappearance of starch early in the ripening process.

ENZYMS IN APPLES

The literature which is available to the writer contains very few references to any investigations of the enzyms that are present in apples.

Lindet (9) found in the juice of apples a soluble ferment which causes coloration of the tissues by the absorption of oxygen and the giving off of carbon dioxid, which is inoperative when the juice has been boiled, which may be precipitated from the juice by alcohol, and which oxidizes pyrogallol to purpurogallin. He concluded that the coloration is due to oxidation of tannin by a soluble ferment of the kind designated by Bertrand as laccase (now called "oxidase").

Warcollier (12) is the only other author who reports work on enzyms in apples. Although he was unable to find invertase in apple juice, he believes that it must be present in order to account for the apparent inversion of sucrose during the ripening process. He suggests that the enzym may have been retained by the apple marc and consequently may have escaped his observation.

The meagerness of the work which has been done along this line is probably due to the fact that the flesh of the apple is not an important element in the physiology of the plant's growth and has little scientific interest to students of plant physiology or biochemistry. But its economic importance and the desirability of knowledge concerning the ripening process as a factor in the storage of perishable fruit products are apparent and, in the writer's opinion, fully justify a thorough study of the subject. The present paper does not constitute an exhaustive report. It does not include, for example, a comparison of enzymic activity of rapidly maturing varieties of apples as contrasted with those which ripen more slowly and, hence, are better keepers. It is believed, however, that the facts here presented will serve as a foundation for such further work as may be found desirable.

EXPERIMENTAL WORK

The apples used in these investigations were secured from an orchardist in the State of Washington and were of varieties known to be good keepers—i. e., slow in ripening in storage.

PREPARATION OF MATERIAL FOR EXAMINATION

The first problem was naturally that of securing an extract of the cell contents of the apple pulp which would contain the enzyms in active form. Since it was not known whether any or all of these enzyms would be diffusible through the cell walls (extracellular), a preliminary mechanical rupturing of the cells or rendering of them permeable by drying, according to well-known methods of technique in enzym study, was necessary. Several methods were tried, as follows:

(1) Whole apples were run through a horse-radish grater and the resulting pulp pressed in an ordinary laboratory hand press. The resulting juice was thick, with small particles of pulp, and attempts were made to clarify it by filtration. These were unsuccessful because of the clogging of the filter by the pectin bodies of the juice.

(2) Apples were rasped and pressed as before and the juice allowed to stand for some time, during which the suspended solids settled fairly well, and the supernatant clear juice was decanted. Precautions against enzymic activity during the settling were taken by keeping the settling jars in an ice box.

(3) An attempt was made to secure a dry powder of the apple pulp by drying thin slices in a vacuum desiccator over sulphuric acid; but the large proportion of sugars and pectin bodies in the tissue made this impossible, the slices being gummy and impossible to grind into a powder even after six weeks' exposure in the desiccator.

(4) Thin slices of apple pulp were treated by the acetone-ether method first used by Buchner, Albert, and Rapp (1) in the preparation of *Dauerhefe*, or active dry yeast powder. This process was very satisfactory, the apple slices, after the treatment and exposure to the air overnight, becoming so dry and brittle that they could easily be powdered between the fingers and very easily reduced to a fine powder in a mortar. Several investigators have reported that the enzymic activity of the dry powder so prepared is not less than that of the original tissue, and the writer's observations confirm this. This appears to afford an excellent means of preparation of sugary or gummy materials of this kind for enzym extractions.

(5) Apples were peeled and cored, and the flesh cut into small blocks. These were then mixed with an equal weight of sharp quartz sand and the mixture rubbed gently in a mortar until uniformly disintegrated. The mixture was then transferred to a fine silk cloth and pressed gently. By this means a limpid juice could be obtained which was nearly free from pectin materials, although slightly cloudy with suspended particles of pulp. Experience has shown that harsh grinding and severe pressure result in diminished activity of the juice, particularly in its oxidase activity, but with gentle manipulation, as above, very active juice can be obtained.

(6) A quantity of concentrated apple juice prepared by Gore (6) by his freezing method was secured and used in some of the tests, since it was thought that this process would be likely to leave the enzyms uninjured in the juice.

EXAMINATION OF DIFFERENT PREPARATIONS FOR ENZYMS

In the earlier examinations reported below, several different preparations were examined simultaneously for the particular type of enzym which was being sought, in order to avoid any wrong conclusion from improperly prepared material. Experience soon showed, however, that either the acetone-dried powder or the pulp ground with quartz sand would yield active extracts in every case where activity could be found in material prepared by any of the above methods, and one or the other of these two preparations was used in all the later tests. The acetone-dried powder has the advantage that a considerable quantity of material can be prepared at one time for subsequent examination.

DIASTASES

Diastases have been shown by Thatcher and Koch (11) to be readily diffusible into water surrounding cell tissues. It seemed probable, therefore, that if enzyms of this type were present in apple flesh they would appear in juice expressed from pulp after thorough rasping. Samples of clear juice by decantation were secured from three different varieties of apples and tested for diastatic activity. Four separate mixtures were prepared for each variety of juice. The first contained 10 c. c. of a 10 per cent solution of soluble starch prepared by the Lintner method (5), 10 c. c. of the juice in question, and 10 c. c. of distilled water. The second contained 10 c. c. of soluble starch, 10 c. c. of the juice which had been boiled for 10 minutes and made to its original volume with water, and 10 c. c. of distilled water. The third contained 10 c. c. of soluble starch, 10 c. c. of the unboiled juice, sufficient *N/10* sodium hydroxid (NaOH) to exactly neutralize the juice used (determined by a preliminary titration, using phenolphthalein as indicator), and enough distilled water to make the total volume 30 c. c. The fourth, or control, contained 10 c. c. of soluble starch and 20 c. c. of distilled water. The contents of each flask were thoroughly mixed and an aliquot drawn off for the determination of reducing sugars present in the solution. The flasks containing the remainder of the solution were then placed in an incubator for 30 minutes at 40° C., these being the conditions recommended by Sherman, Kendall, and Clarke (10) for all determinations of diastatic activity. At the expiration of this period action was stopped by adding sufficient *N/10* sulphuric acid to make the total volume a *N/200* solution, and an aliquot equal to that taken before the digestion was drawn off for the determination of its reducing sugar content. The soluble proteins were precipitated and the reducing sugars determined by the method out-

lined in the article by Thatcher and Koch (11). The results obtained are given in Table I.

TABLE I.—*Results of tests for diastase in the flesh of apples*

Variety and material.	Reducing sugars.	
	Before action.	After action.
	Gm.	*Gm.*
Jonathan:		
Decanted juice	0.0192	0.0183
Decanted juice (boiled)	.0207	.0212
Decanted juice (neutralized)	.0197	.0192
Control (water only)	None.	None.
Yellow Newtown Pippin:		
Decanted juice	.0113	.0113
Decanted juice (boiled)	.0217	.0212
Decanted juice (neutralized)	.0103	.0113
Control	None.	None.
Rome Beauty:		
Decanted juice	.0103	.0098
Decanted juice (boiled)	.0207	.0202
Decanted juice (neutralized)	.0113	.0103
Control	None.	None.

At a later date, when other preparations of apple material were available, tests were made of the reducing sugars present in equal aliquots of soluble-starch solution which had been digested for 30 minutes at 40° C., with both boiled and unboiled extracts of these materials, with the results given in Table II.

TABLE II.—*Results of tests for diastases in various preparations made from the flesh of apples*

Material.	Reducing sugars found after action.	
	Active extract.	Boiled extract.
	Gm.	*Gm.*
Water extract of acetone-dried pulp	0.0202	0.0207
Juice concentrated by Gore's process	.0356	.0351
Juice from pulp ground with quartz sand	.0316	.0316

From these results it is evident that the juice contained no diastases. It appears, therefore, that after the starch disappears from the apples the diastases also disappear. None of the apples which were available for these investigations contained any starch.

INVERTASE

Invertase was tested for in two samples by a method precisely like that used for diastases except that 10 c. c. of a 10 per cent solution of sucrose were used in place of the soluble starch. The results obtained are given in Table III.

TABLE III.—*Results of tests for invertase in the flesh of apples*

Variety and material.	Reducing sugars.	
	Before action.	After action.
	Gm.	*Gm.*
Yellow Newtown Pippin:		
Decanted juice	0.0113	0.0113
Decanted juice (boiled)	.0212	.0217
Decanted juice (neutralized)	.0113	.0103
Control (water only)	None.	None.
Rome Beauty:		
Decanted juice	.0098	.0103
Decanted juice (boiled)	.0202	.0207
Decanted juice (neutralized)	.0103	.0113
Control	None.	None.

These results being so contrary to what was expected, it was thought best to use material prepared for examination in several other ways in testing for invertase. Accordingly, a water extract was made of some acetone-dried powder from Rome Beauty apples, another sample of the same apples was ground with quartz sand and its juice expressed, and finally a sample of the Gore's concentrated apple juice was diluted to about the same concentration as normal apple juice. Each of these materials was then incubated with sugar solution in the usual way, using unboiled and boiled samples of both the acid and neutralized juice in each extract. The reducing sugars found in the digested mixture from the unboiled or "active" extract and from an equal aliquot of boiled extract are given in Table IV.

TABLE IV.—*Tests for invertase in various preparations from the flesh of apples*

Material.	Reducing sugars found after action.	
	Active extract.	Boiled extract.
	Gm.	*Gm.*
Water extract of acetone-dried pulp	0.0207	0.0207
Juice concentrated by Gore's process	.0396	.0396
Juice concentrated by Gore's process (neutralized)	.0376	Lost.
Juice from pulp ground with quartz sand	.0192	.0187
Juice from pulp ground with quartz sand (neutralized)	.0192	.0192

The results shown in Tables III and IV indicate the absence of any invertase in apple flesh and confirm the observations of Warcollier (12), referred to above. It appears, therefore, that changes during ripening which result in the inversion of sucrose, if they actually occur, must be due to some other cause than the presence of invertase in the apple tissues. The fact that some investigators have not been able to find evidence of this inversion of sucrose during ripening casts some doubt upon its actual

occurrence, there being always the possibility that observed changes in the nature of the sugars present in successive samples may be due to the action of organic acids during the preparation of the samples for analysis.

TANNASE

Determinations of the tannin content of each of the four varieties of apples which were being used by Proctor's modification of Löwenthal's method[1] showed that the flesh of the apples contained the following percentages of tannin: Rome Beauty, 0.208; Arkansas Black, 0.192; Yellow Newtown Pippin, 0.208; King David, 0.132.

It seemed advisable to ascertain, therefore, whether any tannin-hydrolyzing enzym was present in these tissues. Accordingly, a quantity of pulp from each variety was ground with quartz sand and the juice expressed. One portion of the juice from each variety was boiled and another left unboiled. Aliquots of the boiled and unboiled juice were placed in each of two test tubes, to one of which 2 c. c. of a 10 per cent solution of Merck's tannic acid was added, in order to insure sufficient excess of substrate material. The four sets of four tubes each were placed in an incubator at 40° C. for 24 hours. At the end of this time a few drops of a 10 per cent solution of ferric chlorid were added to each test tube and the intensity of color developed in the tubes containing check boiled and unboiled juices was compared. In no case could the slightest difference in intensity of color be observed, from which it was concluded that the juices contained no tannase.

EMULSIN

Glucoside-splitting enzyms were tested for in boiled and unboiled juices prepared from each of the four varieties of apples by digesting aliquots of these juices with 2 c. c. of a 1 per cent solution of amygdalin for 24 hours at 40° C. In no case was any odor of benzaldehyde perceptible at the end of this time, while check tubes to which emulsin was added gave a pronounced odor after only 10 minutes' contact with the amygdalin used. Hence, it was concluded that the apple flesh contains no enzym of the emulsin type.

ESTERASES

One of the noticeable changes in an apple during the ripening period is the development of its characteristic odor and flavor, due chiefly to the ester ethyl malonate. Such esters are usually accompanied in nature by a corresponding esterase; hence, it seemed advisable to test the flesh of the apples for an esterase which would hydrolyze ethyl malonate.

Accordingly, apple juice was obtained by the quartz-sand method and a series of test tubes prepared with the following contents: (1) 5 c.c. of apple juice, 5 c. c. of ethyl malonate, and 10 c. c. of distilled water; (2) 5 c. c. of apple juice which had been previously boiled for 10 minutes,

[1] Wiley, H. W., et al. Official and provisional methods of analysis, Association of Official Agricultural Chemists. U. S. Dept. Agr. Bur. Chem. Bul. 107 (rev.), 272 p. 1908. See p. 150.

cooled, and made up to its original volume, 5 c. c. of ethyl malonate and 10 c. c. of distilled water; (3) 5 c. c. of apple juice, 5 c. c. of ethyl malonate, sufficient *N/10* sodium hydroxid to render the mixture alkaline in reaction, and enough distilled water to make the total volume the same as in the other tubes; (4), (5), and (6) the same as (1), (2), and (3), respectively, except that a 0.1 per cent solution of steapsin was used in place of the apple juice, as a check upon the reaction conditions. These mixtures were kept in an incubator at 40° C. for 20 hours, after which an aliquot of the mixture was drawn off and titrated with *N/100* sodium hydroxid, using phenolphthalein as indicator, with the results given in Table V.

TABLE V.—*Test for esterases in the flesh of apples*

[Ethyl malonate used as substrate]

Material.	N/100 alkali required.
	c. c.
(1) Apple juice	9.2
(2) Apple juice (boiled)	7.5
(3) Apple juice (with excess of *N/10* alkali)	[a]39.8
(4) Steapsin solution	7.3
(5) Steapsin solution (boiled)	None.
(6) Steapsin solution (with excess of *N/10* alkali)	40.9

[a] In addition to *N/10* sodium hydroxid used to make reaction alkaline.

The data presented in this table clearly indicate the presence in the juice of an esterase capable of hydrolyzing ethyl malonate and similar in its action to steapsin. A slight increase of acidity in test tube (1) over that in (2) indicates a slight hydrolytic action even in the acid medium of the unneutralized juice; while in alkaline medium the activity was almost identical with that of the 0.1 per cent steapsin acting in a similar medium.

OXIDASES

Owing to the fact that Lindet's observations (9) mentioned above, the well-known phenomenon of the coloring of apple tissues when exposed to the air, and the qualitative guaiac reaction for oxidases all point to the presence of active oxidases in apples, a quantitative determination of their presence in the different samples available for this investigation was determined upon. Bunzel (3) has shown the objections to the various methods which have been proposed for the quantitative measurement of oxidase activity by various colorimetric determinations and has perfected a manometric method for the purpose. Correspondence with Dr. Bunzel resulted in his kind permission to make use of his apparatus for the investigation of the materials used in this study. Several samples were accordingly taken to his laboratory and their action toward various oxidizable materials determined according to his method. In carrying out the operation, 0.1 gm. of the acetone-dried powder or 2 c. c. of the apple juice obtained by the quartz-sand method were intro-

duced into one arm of the apparatus, 0.01 gm. of the material to be oxidized placed in the other arm, the proper amount of distilled water added in each arm, and the apparatus placed in the constant-temperature box and allowed to stand for 30 minutes to come to a uniform temperature. The apparatus was then closed, the shaking started, and the manometer readings taken at 15-minute intervals. The final readings, with the kind of material and nature of oxidizable reagent used in each case are given in Table VI.

TABLE VI.—*Oxidase activity of various apple preparations toward different oxidizable reagents*

Variety and material.	Oxidizable reagent.	Time of maximum action.	Diminished pressure.
		Min.	*Cm.*
Rome Beauty:			
Acetone-dried powder	Pyrogallol	45	0.10
Do	Pyrocatechol	60	.60
Do	Guaiacol		0
Do	Tyrosin		0
Yellow Newtown Pippin:			
Acetone-dried powder	Pyrogallol	45	.35
Do	Pyrocatechol	60	1.75
Do	Guaiacol	60	.15
Do	Tyrosin		0
King David:			
Acetone-dried powder	Pyrogallol	60	.20
Do	Pyrocatechol	60	1.45
Do	Guaiacol	60	.15
Do	Tyrosin		0
Arkansas Black:			
Acetone-dried powder	Pyrogallol		0
Do	Pyrocatechol	45	.55
Do	Guaiacol		0
Do	Tyrosin		0
Juice from pulp with quartz sand	Pyrogallol	30	1.45
Do	Pyrocatechol	30	3.50
Juice from pulp with quartz sand(boiled)	Pyrogallol		0
Do	Pyrocatechol		0

These results clearly show that apple pulp and apple juice contain an active oxidase, or oxidases, which accelerate the absorption of atmospheric oxygen by pyrocatechol and pyrogallol, and to a slight extent by guaiacol. The activity toward pyrocatechol is much greater than toward the other reagents, indicating the probability that the tannin of apples, which is so readily oxidized on exposure to air under the influence of the oxidases present, is of the pyrocatechol type.

PROTEASES

Protein-splitting enzyms in the flesh of the apple were tested for as follows: A saturated solution of egg albumin was prepared and 5 c. c. of it were placed in each of three test tubes. In one of these, 5 c. c. of apple juice, prepared by grinding the pulp with quartz sand, were added; to the second, 5 c. c. of the same juice, which had been boiled for 10 minutes,

cooled, and made to its original volume; and to the third, 5 c. c. of distilled water. Another set of three test tubes was prepared with the same proportions of materials, but using a 1 per cent solution of Witte's peptone in place of the albumin solution. The tubes so prepared were kept in an incubator at 40° C. for 24 hours. At the end of this time an aliquot of each mixture was drawn off and the quantity of amino acids present in it determined by the ninhydrin method recently proposed by Harding and MacLean (7), using a solution of glutamic acid containing the equivalent of 0.1 mgm. of nitrogen in the amino-acid form per cubic centimeter for the production of the standard color.

The characteristic color due to amino acids appeared in all the tests except the one in which only water and albumin were used. The amino-acid equivalent in each case, as determined by comparison with the standard color, is given in Table VII.

TABLE VII.—*Tests for proteases in the flesh of apples*

Material.	Amino-acid equivalent after action (milligrams of nitrogen).
Unboiled juice + egg albumin	0.12
Boiled juice + egg albumin	.07
Water + egg albumin	None.
Unboiled juice + peptone	.10
Boiled juice + peptone	.10
Water + peptone	.03

It appears from these data that both the juice itself and the peptone used contained amino acids which would give a blue color with the ninhydrin reagent. But the incubated mixture of unboiled juice and albumen contained more amino acids than that in which an equal volume of boiled juice was used; while with peptone no increase of amino acid was produced by the unboiled juice, and the total amino acid found was just equal to the sum of that found in the quantity of juice and of peptone solution used in the tests. It thus appears that the juice extracted by grinding with quartz sand contains a small amount of some protein-splitting enzym of the trypsin or papain type rather than of the erepsin type. It was concluded, therefore, that the flesh of the apples contains a small amount of protease, to the action of which on the protein material of the apple cells is due the small amount of amino acid found to be present in the juice of the ripening fruit.

PECTINASES

The fact that the flesh of an apple softens and becomes mealy or mushy at the close of the ripening period is generally attributed to the solution of the middle lamella and the consequent separation of the cells of the tissues. The solution of the middle lamella is supposed to be the work of an enzym known as pectinase. It is supposed, therefore, that pecti-

nase occurs in ripening fruits. It was intended at the outset to ascertain whether a pectinase was present in the apples used in this investigation, but review of the literature dealing with methods of detection of pectinase, as summarized by Cooley (4) in a recent article, together with the unsatisfactory results of Cooley's own use of these methods in testing for pectinase in diseased plums, made it appear doubtful that accurate evidence on this point could be secured. Some preliminary tests of the methods which had been suggested confirmed the writer's opinion in this respect, and the attempts were postponed until such time as more satisfactory methods of testing for pectinases have been devised.

ENZYMS IN THE SEEDS OF APPLES

Although the occurrence of different enzyms in the seeds of the apple would not have any bearing upon the ripening processes in the flesh of the apple and, hence, is of no importance to the particular object of this investigation, such an excellent opportunity was offered to test for enzyms in the seeds at the same time that the tests were being applied to the flesh or juice, that it was determined to carry on such a series of tests. Accordingly, a large number of seeds, some 20 gm. in all, were picked out of several apples, and the brown seed coat was picked from each seed. The white seeds were then kept for about two weeks in a vacuum desiccator until they were dry enough so that when crushed they gave off no odor of benzaldehyde, thus indicating that not enough water was present to permit the glucosidase action to occur.

A weighed quantity of the dry seeds was then ground in a mortar with sharp quartz sand until the seeds were thoroughly disintegrated. The material was then preserved in a tightly stoppered weighing bottle until needed for each test. For the tests, 2 gm. of the mixture, equivalent to 1 gm. of dry seeds, were digested at room temperature for 30 minutes with 100 c. c. of distilled water, and a filtered aliquot of this extract was used for the tests. A detailed description of the progress of each particular test is unnecessary in this article, but the results obtained, based upon a comparison of unboiled and boiled extracts with water controls, show the following facts with reference to the presence of the various enzyms which were tested for in apple seeds: Diastases, present in considerable amount; invertase, absent; emulsin, present in considerable amount; lipase, present in small amount; protease, present (hydrolyzes both albumin and peptone); oxidases, absent.

SUMMARY

From the results of these investigations it appears that the only enzyms which participate in the changes in the carbohydrates of apples during the ripening process are oxidases. None of the common types of carbohydrate-splitting enzyms could be found. The fact that the changes which take place during ripening are inhibited by surrounding the fruit in an atmosphere of carbon dioxid, as shown by the experiment described

in the opening paragraphs of this article, is easily explained on the basis of their being oxidase changes, since it is a well-known fact in enzymology that the presence of a large excess of the end products of a reaction generally inhibits the action of the accelerating enzym in increasing degree as the proportion of the end product increases. Carbon dioxid is undoubtedly the end product of oxidase activity and should therefore accomplish just the result which was found to occur in the jar in which this gas was used.

The small amounts of esterase and of protease which were found in the ripening apples indicate the possibility of the hydrolytic decomposition of the small quantity of essential oil and of protein material contained in the flesh of the apple during the ripening process or subsequent breaking down of the tissue.

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AN AUTOMATIC TRANSPIRATION SCALE OF LARGE CAPACITY FOR USE WITH FREELY EXPOSED PLANTS

By LYMAN J. BRIGGS, *Biophysicist in Charge, Biophysical Investigations*, and H. L. SHANTZ, *Plant Physiologist, Alkali and Drought Resistant Plant Investigations, Bureau of Plant Industry.*

INTRODUCTION

An extended study of the transpiration rate of plants practically necessitates the use of an automatic balance of some type. The present paper contains a review of the various forms of transpiration balances heretofore employed, together with a description of a new automatic transpiration scale of large capacity, so designed that the plants may be freely exposed to the weather. Four of these scales have been in continuous use during the past four summers at Akron, Colo.

Automatic balances may be divided into two classes: (1) The step-by-step type, in which small weights of equal value are added to the scale pan in succession or a counterpoise is advanced in equal steps; (2) the continuous record type, in which the plant is suspended from a spring or from a variable lever or is mounted directly on a float.

RECORDING BALANCES OF THE STEP-BY-STEP TYPE

Vesque (1878)[1] appears to have been the first to employ an automatic balance in measuring transpiration. He made use of the step-by-step principle, a measured quantity of mercury being delivered to a receptacle on the scale pan each time the beam tipped sufficiently to close an electric circuit. His apparatus is illustrated in figure 1, the device for measuring the mercury being shown at *s* and enlarged at B. This measuring device is in principle similar to a large stopcock, in which the plug is only partially bored through from each side so as to form two shallow cavities of equal volume. Either cavity in its upper position becomes filled with mercury from the reservoir *t*. When the circuit is closed, a spring motor is released, which turns the plug through one-half a revolution, delivering the mercury in the cavity to the container *a*, and recording the time of the event by lowering the stylus *p* in contact with the circular plate *v* of the clock H.

Anderson (1894) was the first to employ steel balls of uniform size as weights for a recording balance. The balls were held in a spiral brass tube, with a block at the lower end containing a pocket for one ball. When the balance beam tipped sufficiently to close an electric circuit, the block was moved sidewise and the ball in the pocket dropped into the

[1] Bibliographic citations in parentheses refer to "Literature cited," p. 131–132.

Journal of Agricultural Research, Dept. of Agriculture, Washington, D. C.

Vol. V, No. 3
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pan of the balance (fig. 2). The weight thus added opened the circuit, and a spring restored the block to its normal position, where the pocket was again filled by a ball from the reserve supply. Anderson did not

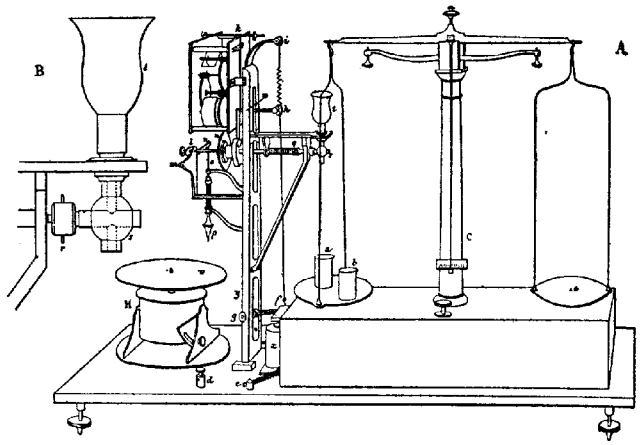

FIG. 1.—Vesque's automatic balance for measuring transpiration. In this apparatus measured quantities of mercury are added to the receiver on the balance pan to counterbalance the transpiration losses.

place the plant directly on the balance, but used his apparatus to register the gain in weight of absorption tubes connected with the transpiration chamber. He does not describe the form of the recording apparatus employed.

Ganong (1905) in his "autographic transpirometer" (fig. 3) combined the ball-dropping and the recording mechanism in a compact and con-

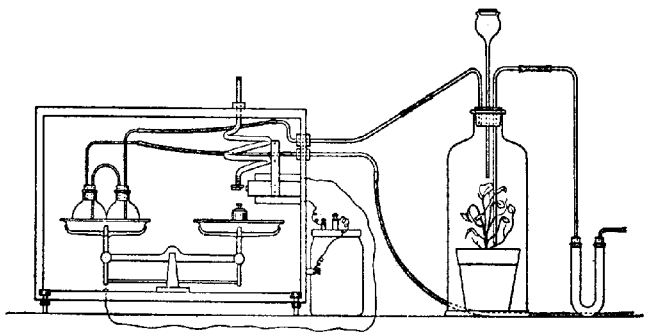

FIG. 2.—Anderson's apparatus for measuring transpiration, in which steel balls are used as weights.

venient form, one electromagnet serving both purposes. Steel balls one-fourth of an inch in diameter were employed as weights. Balls of this size approximate 1 gm. each in weight. The clock was so arranged

that by offsetting the cylinder daily a weekly record could be obtained on one sheet.

Transeau (1911), in working with xerophytes, employed hollow brass balls standardized to 0.4 gm. in place of ¼-inch steel balls of 1 gm. weight, but states that the hollow balls are not as light as could be desired. The writers have found that ⅛-inch steel balls weighing 0.13 gm. can be readily used, provided the valve [1] is constructed to fit them.

Woods (1895) used the automatic weighing rain gage of Marvin (1903) as a transpiration balance, the apparatus being modified to record loss instead of gain in weight (fig. 4). In this apparatus the counterpoise is

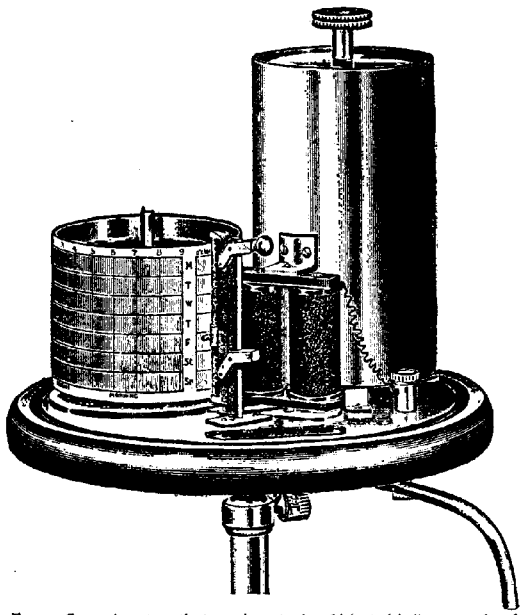

FIG. 3.—Ganong's automatic transpirometer in which steel balls are employed as weights.

moved along the beam in $\frac{1}{10}$-gm. steps by a screw actuated by an electromagnet carried on the balance itself. The recorder (fig. 5) is independent of the balance.

Blackman and Paine (1914) have recently described a recording transpirometer operating on the step-by-step principle, in which "water drops are used in place of steel balls, the water being added directly to the soil." Their apparatus has been represented schematically in figure 6. Water is allowed to drip continuously from a Mariotte system. During the greater part of the time the drops are intercepted by a movable

[1] For description of valve, see under "Ball-dropping device," p. 123.

funnel and collected as waste water. When the plant through transpiration causes the balance beam to tip sufficiently to close an electric circuit, the funnel F is withdrawn by the solenoid A, and the water drops fall directly into a receiving tube leading to the soil in the pot. Water is thus added directly to the pot until the balance tips sufficiently in the opposite direction to close a circuit through a second solenoid B, which restores the funnel to its intercepting position. The time at which the circuit is closed is electrically recorded on a clock drum. The position of the contacts is adjustable, so that the quantity of water added each time—i. e., the size of the steps—may be modified to suit the transpiration rate. This method is unique and advantageous in maintaining the moisture content of the soil constant throughout the experiment. Under freely exposed conditions, however, the quantity of water added each time would be variable and indeterminate, due to the oscillation of the balance by the wind.

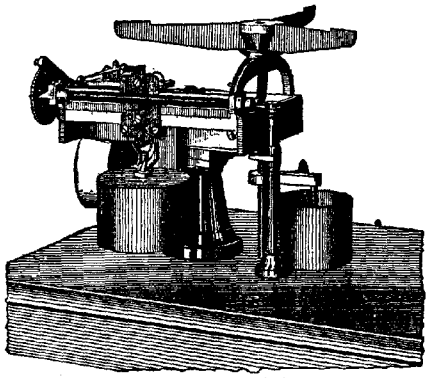

FIG. 4.—Woods' adaptation of Marvin's weighing rain gage as a transpiration balance. In this apparatus the loss through transpiration is counterbalanced by a weight controlled by a screw.

TRANSPIRATION BALANCES OF THE CONTINUOUS-RECORD TYPE

The first continuously recording transpiration apparatus appears to have been devised by Krutizky (1878). It is of interest to note that the first step-by-step recording apparatus was described by Vesque in the same year. Krutizky's apparatus is shown in figure 7. The water lost through transpiration from a potometer is continuously replaced through a siphon from a supply con-

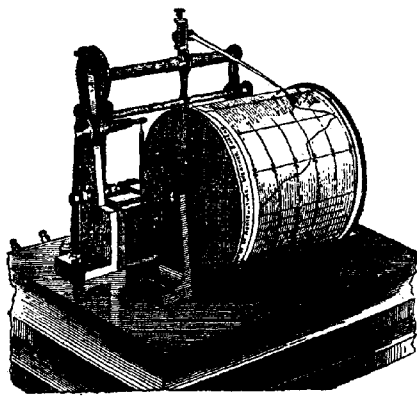

FIG. 5.—The Marvin register used by Woods for recording transpiration.

tained in a floating cylinder *a*, which rises as the load decreases and traces its movement on the smoked drum of a clock. Like other apparatus involving the principle of flotation, this apparatus is subject to errors arising from changes in buoyancy accompanying changes in temperature.

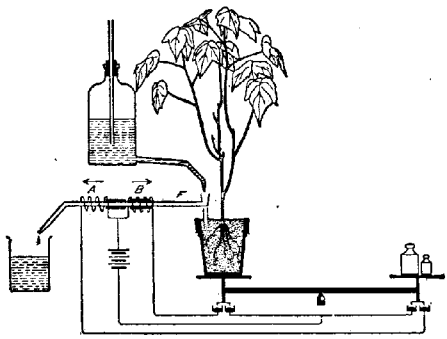

FIG. 6.—Schematic diagram of Blackman and Paine's recording transpirometer, in which water is automatically added to the pot to offset the transpiration loss, so that the moisture content of the soil is kept uniform.

A transpiration balance devised by Richard Frères (Burgerstein, 1904, p. 8–9) is illustrated in figure 8. The balance is made very insensitive by a heavy bob. The movement of the balance pan from the "down" to the "up" position corresponds to a known loss in weight, depending on the weight and position of the bob. The movement of the beam is recorded directly on the drum of a clock.

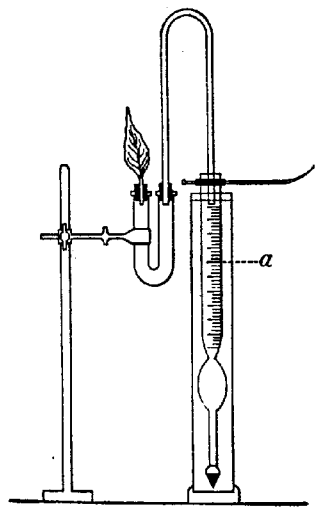

FIG. 7.—Krutizky's potometer for recording transpiration, in which the loss from the potometer is continuously replaced from the supply in the floating cylinder.

Copeland (1898) has described an apparatus (fig. 9) for recording transpiration in which the weight of the plant is balanced over a pulley by the weight of a partially submerged hydrometer bulb. The pulley shaft rolls on plate-glass supports to reduce the friction. A tracer supported from a second wheel records the motion on smoked paper on a clock cylinder. With its maximum load (3.5 kgm.) the instrument responds to a change in weight of 0.05 gm.

Corbett (1900) has used a large Nicholson hydrometer for measuring transpiration, the plant being placed directly on the pan *a* of the hydrometer *b* (fig. 10). The apparatus is made self-recording by connecting the float with the lever of an auxanometer. This apparatus, like that of Copeland, is affected by temperature, which changes the density of the water and consequently

its buoyancy. Temperature effects can, however, be practically eliminated by surrounding the hydrometer tank with a water-jacket, through which water is constantly circulating. The sensibility of the apparatus is determined by the cross section of the stem of the hydrometer.

A NEW AUTOMATIC TRANSPIRATION SCALE OF LARGE CAPACITY

The requirements of the transpiration studies at Akron necessitated an automatic weighing apparatus having a carrying capacity of 150 kgm., capable of operating positively in the wind, and so designed that the plants could be freely and continuously exposed to the weather (Pl. IX). A platform scale with agate bearings having a carrying capacity of 200 kgm. and a sensibility of 5 gm. was chosen for equipment as an automatic balance of the step-by-step type (Pl. X). The scale was fitted with a short column so as to bring all the mechanism below the level of the top of the pot and was provided with the following auxiliary equipment:

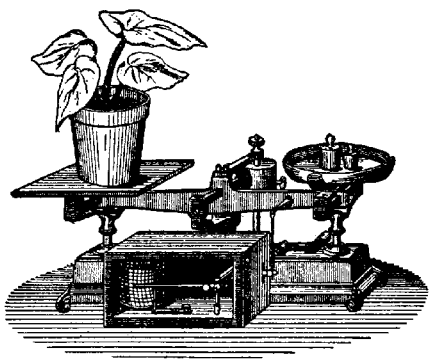

FIG. 8.—The transpiration balance of Richard Frères with its recording apparatus.

a. Ball-dropping device.
b. Ball receiver on beam.
c. Beam contact and mercury cups.
d. Oil dashpot on beam.
e. Spring motor for raising beam.
f. Adjustable counterpoise for raising the center of gravity of balanced system.
g. Recorder for registering time at which each ball is dropped.
h. Batteries and relay.
i. Case for protecting mechanism from the weather.

The beam of the scale with a part of the auxiliary equipment is shown in fig. 11. The operation of the mechanism is briefly as follows: As the plant decreases in weight, the beam falls until an electric contact is made at U. This closes a relay circuit, with the following results:

1. The ball-dropping device A deposits a ball in the receiver L. The weight of this ball tends to raise the beam.
2. The spring motor, by means of a cam K, raises the beam promptly and positively to its upper position.
3. The time of the event is indicated on the drum of the recorder.

BALL-DROPPING DEVICE.—The ball-dropping device used in our experiments is shown in fig. 12. A commercial telegraph sounder provides an efficient mechanism for actuating the valve. When the circuit is closed, the slide A moves in the direction of the arrow and releases the lowest ball in the tube. The remaining balls are prevented from passing down the tube by the upper septum B, which moves into the tube as the lower septum C moves out. When the circuit is broken, a spring restores the valve mechanism to its original position and the reserve balls slide down the tube so as to rest against the lower septum. The mechanism is now in position to drop another ball as soon as the circuit is again closed.

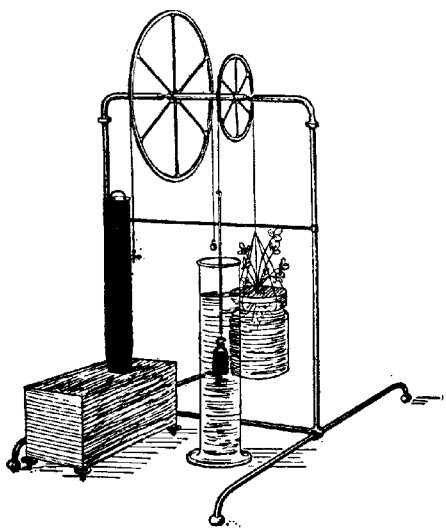

FIG. 9.—Copeland's apparatus for recording transpiration in which the loss in weight through transpiration is counterbalanced by a change in position of a partially submerged float.

As the discharged ball leaves the valve it drops into the balanced receptacle D, which tips downward under the weight of the ball, closing the circuit of the recorder through the mercury cups E below. The ball in the meantime rolls into the funnel and is delivered into the ball receiver I, suspended from the balance beam. With this arrangement no record is made unless the ball is actually received in D, and a second ball can not be recorded until the first has been delivered and D has returned to its initial position. In very gusty weather there is occasionally a fluttering of the valve A, two balls being dropped in rapid succession. The second ball simply shoots over D into the waste cup and is not recorded.

The tube holding the reserve supply of balls (fig. 11) is of glass bent into the form of an open spiral, and is joined to the valve tube by a conical adapter. The diameter of the valve tube at the septa must be only slightly greater than the diameter of the balls to insure the valve's working properly, and the tube should taper gradually to this diameter. The distance between the adjacent faces of the two septa should also be equal to the diameter of the ball. Each septum when in its intercepting

position should extend into the tube approximately one-fourth of the tube diameter. It is essential that the valve be accurately made to conform to the particular size of ball used as a weight. The inside of the valve tube should be kept smooth and clean by the occasional use of benzine, and the balls should also be kept polished.

The balls used for weights were three-sixteenths of an inch in diameter and of first-quality hardened steel. They were found to be so nearly uniform in weight that no appreciable error is introduced by assuming them equal. The individual weights in milligrams of 10 balls selected at random were as follows: 437.0, 438.5, 437.2, 437.7, 436.8, 437.6, 437.3, 438.0, 437.5, 437.0. Mean, 437.4. Probable error for a single ball, 0.4 mgm., or 1 part in 1,000.

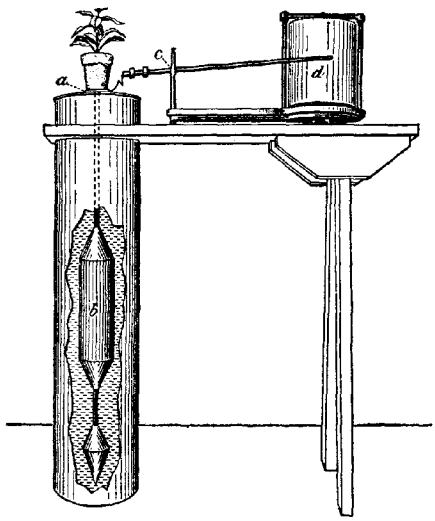

FIG. 10.—Corbett's apparatus for measuring transpiration in which the plant is carried on the pan of a large Nicholson hydrometer.

BALL RECEIVER.—The conical receiver L for the balls is suspended from an extension of the beam (fig. 11) on the same side as the load, since the added weight of the ball compensates for the loss by transpiration. The receiver is suspended from a knife-edge which lies in the plane determined by the two other knife-edges on the beam. The distance from the central knife-edge is so chosen that the weight of a ball corresponds to a change of 20 gm. in the weight on the scale platform.

The measuring tray shown in Plate XI affords a rapid means of counting the balls dropped during any period without touching them. Each complete row includes 10 balls, and the rows are graduated accordingly on the margin. It is essential that the lower end of the tray be cut obliquely so as to form an angle of 60° with the graduated side.

DASHPOT.—The oil dashpot (fig. 13) is an essential part of the apparatus when the balance is exposed to the wind. The resistance can be adjusted to some extent by turning the perforated plate on the top of the inner cylinder I. The outer cylinder O is mounted directly below the weight support on the beam, to which the inner cylinder is attached by the rod N. (See fig. 11.)

SPRING MOTOR FOR RAISING BEAM.—The dropping of a ball into the receiver is ordinarily sufficient to raise the opposite end of the beam and open the circuit. It sometimes happens, however, when the transpiration rate is high and a gusty wind is blowing, that the beam remains down until the transpiration has been sufficient to require a second ball

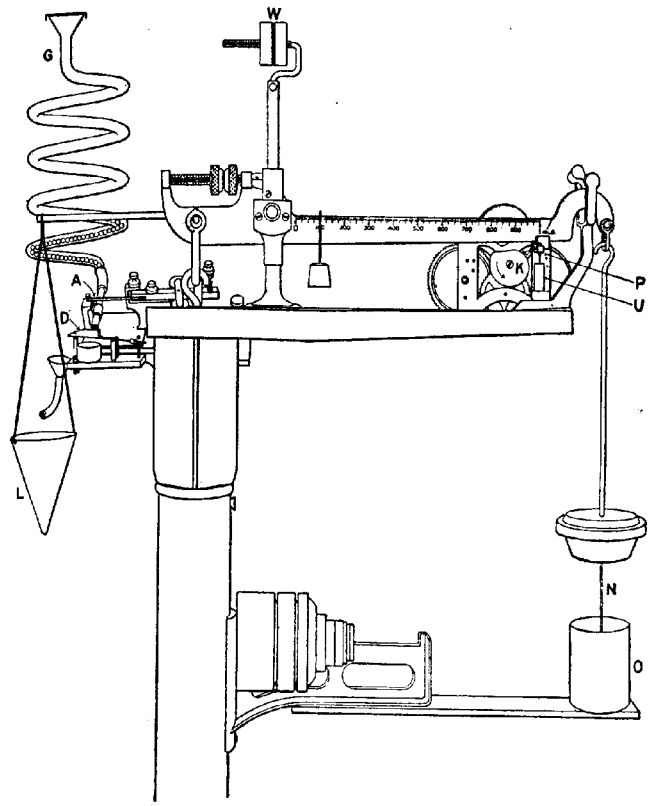

FIG. 11.—View of the beam and auxiliary equipment of the platform transpiration scale designed to carry large pots of plants weighing 150 kgm. or more. As the plant loses weight, the beam falls and the platinum point P closes a circuit through the mercury cup U. This actuates the ball dropper A, which deposits a ball in the receiver L. At the same time the cam K makes one revolution, raising the beam to its upper position and leaving it free to fall. An oil dashpot is provided at O.

to counterbalance the loss in weight. Under such conditions the balance would fail to operate without the intervention of some protective device. This protection is secured by a spring motor which raises the beam to its upper position each time a ball is dropped and then leaves the beam free. The motor, which consists of a strong 8-day clock movement equipped with a fan, F (fig. 14), to reduce the speed, is controlled by

an electromagnet, M (fig. 15).. When the beam circuit is closed, the motor is released and raises the beam through a cam, K (fig. 14). When the cam shaft S (fig. 15) has completed one revolution, the arm H on the cam shaft again engages the spring R on the armature T of the magnet, and the motor is stopped.

ADJUSTABLE POISE FOR RAISING CENTER OF GRAVITY OF BEAM.—It is essential that the mercury contact on the beam be closed with a positive motion to avoid the fluttering of the relay armature. This is accomplished by raising the center of gravity until the beam is slightly unstable, by means of an adjustable bob, W, located above the central knife-edge. (See fig. 11.)

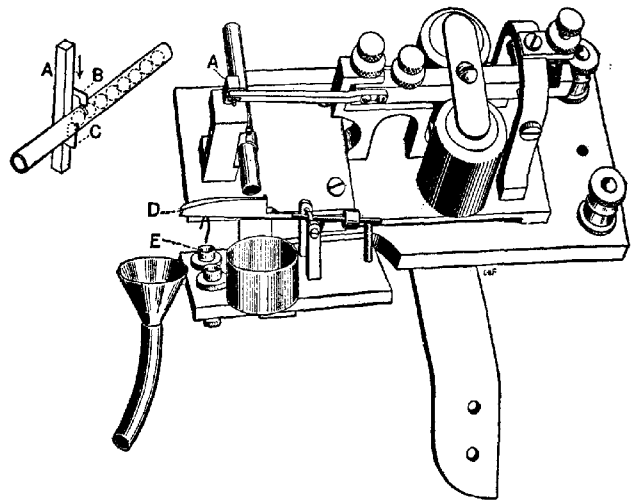

FIG. 12.—Details of the ball-dropping mechanism. The steel ball passes through the valve A into the tipping bucket D, which falls under the weight of the ball and closes an electrical circuit at E to the register.

MARVIN RECORDER.—A convenient type of recorder for registering the time at which each ball is delivered is that devised by Marvin for use in connection with automatic rain gages. This recorder has a drum, 12 inches in circumference, which makes one revolution in six hours and is continously offset by a screw, so that the four 6-hour periods are recorded side by side on the same sheet. A valuable feature is a zigzag attachment on the magnet, by means of which the tracing pen is permanently displaced each time the magnet circuit is closed. This gives a record which is much easier to read than the ordinary record in which the pen returns to its initial position when the circuit is opened (fig. 16). The dropping of two balls in rapid succession is easily seen in the zigzag

record on account of the double offset, but is difficult to determine in a record of the ordinary type.

PROTECTING CASE.—A tight weatherproof case inclosing the column and beam of the balance protects the automatic equipment from the

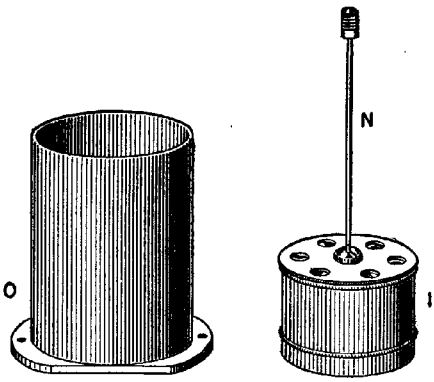

FIG. 13.—Dashpot for preventing the oscillation of the beam during windy weather.

weather. The case is equipped with a removable top and a sliding front. The latter is also supplied with a smaller door through which the apparatus can be observed and adjusted.

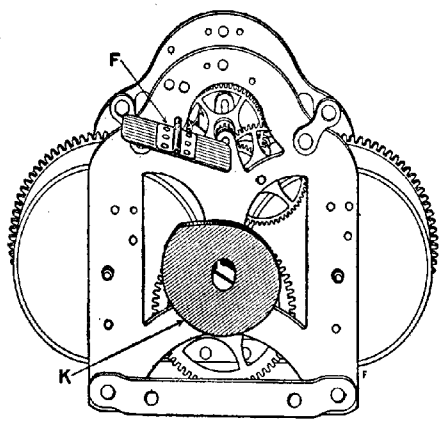

FIG. 14.—Spring motor, showing the cam K for raising the beam, and the fan F for regulating the speed.

ELECTRIC CIRCUITS.—The electrical connections consist of three circuits (fig. 17). A single dry cell, B_1, operates the relay through the beam contact. The ball valve and the motor release are connected in parallel in a second circuit, B_2, containing a battery of three or four cells. This

circuit is controlled by the relay contact. The recorder is operated by a third circuit, B_3, controlled by the tipping bucket on the ball valve. Each circuit is closed only momentarily, and the dry cells usually need to be renewed but once during the summer.

AUTOGRAPHIC RECORDS FROM THE AUTOMATIC TRANSPIRATION SCALE

The results of our transpiration measurements will be presented in other papers, but it seems desirable to reproduce here several daily records illustrating the actual performance of the apparatus. A word of explanation in connection with the interpretation of the records may be helpful. The clock drum makes four revolutions during the day, so that the record is divided into four 6-hour periods. The pen is offset at the moment each ball is delivered. There are five such offsets or steps in one direction (up, for instance) and then five steps in the opposite direction. Since each offset corresponds to a loss of 20 gm. of water, the interval from the crest to the trough of the graph is the time required for the transpiration of 100 gm. of water, or from crest to crest, the time interval for 200 gm. loss.

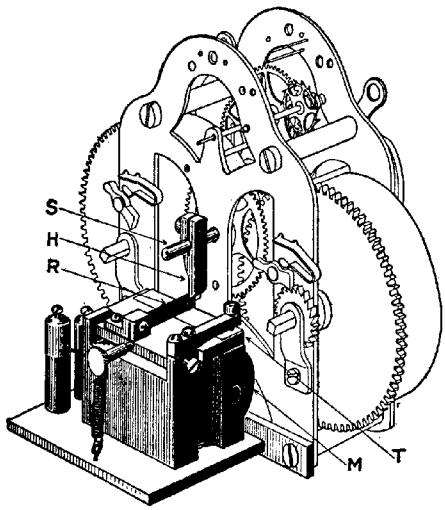

FIG. 15.—Another view of the spring motor, showing the control mechanism. When the magnet M is energized, the spring R attached to the armature T is pulled down, releasing the motor. Raising the beam de-energizes M, so that the motor, after making one revolution, is stopped by H again coming in contact with R.

The wheat records shown in figure 16 were taken from a series obtained in 1912 inside the screened inclosure used in the water-requirement experiments. The normal wind velocity was reduced somewhat by the inclosure and by the proximity of other plants. The first record reproduced (July 2, 1912) was obtained on a clear day. It will be noted that the time interval shortens as midday is approached—that is, the transpiration rate increases and attains its maximum value about 3 p. m., after which it falls rapidly. The transpiration at night, represented by

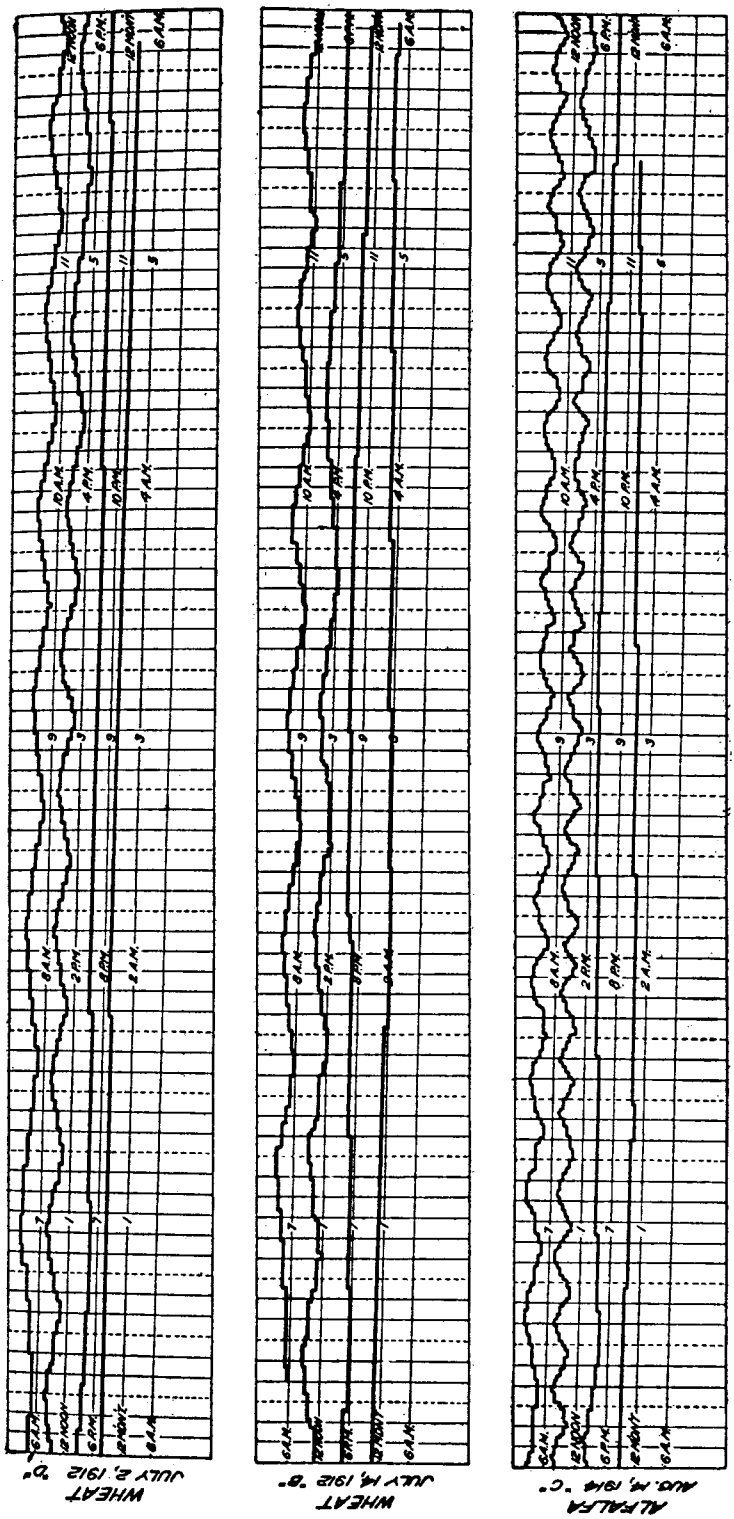

FIG. 16.—Sample records from the automatic transpiration scale. Each step corresponds to a transpiration loss of 20 gm., or 100 gm. from crest to trough of the graph.

the two lower lines of the graph, is seen to be very small as compared with the day transpiration.

The second graph for wheat (July 14, 1912) was selected to show the effect of cloudiness in the afternoon, beginning at 3.30 p. m. The change in the transpiration rate is seen to occur soon after this, and the transpiration between 5 and 6 p. m. is very low compared with that on a clear day, as shown by the first chart. The transpiration during the night of July 14 was higher than during the night of July 2. Automatic measurements with a wet-bulb instrument show that the air contained less moisture during the night of July 14 than during the night of July 2, which would account for the increased transpiration. The temperature on the two days was practically the same.

The third chart shows a record of a pot of alfalfa, taken outside the inclosure. The plants were freely exposed to the wind, which ranged in velocity from 7 to 14 miles per hour during the morning and from 2 to 5 miles during the afternoon. Over 8 liters of water were transpired

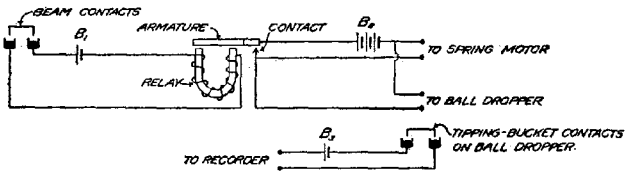

FIG. 17.—Wiring diagram of the electric circuits of the automatic transpiration scale.

during the day, and it is of interest to note how closely this loss is confined to the daylight hours.

The transpiration recorded on the three record sheets reproduced in figure 16 is plotted in rectangular coordinates in figure 18, showing for each pot of plants the transpiration rate in grams per hour for each hour of the day. It may be added that the pots used were equipped with sealed covers, so that the loss of water by direct evaporation from the soil was practically eliminated.

SUMMARY

This paper describes an automatic transpiration scale of 200 kgm. capacity and 5 gm. sensibility, designed for use in connection with the large culture pots employed by the writers in water-requirement measurements. The apparatus is so constructed that the plants may be freely exposed to wind and weather. Steel balls are used as weights, as in Anderson's balance, each ball corresponding to a change in weight of 20 gm. A spring motor is provided to lift the beam positively when a ball is dropped, which is an essential feature when plants are exposed to wind. The apparatus works very satisfactorily except in the presence of whirlwinds or sudden gusts, which lift the plants and tend to give a transpiration

rate which is momentarily too high. Special provision is made to prevent two balls being delivered to the beam in rapid succession, and no record is made unless a ball is actually delivered to the ball container on the beam. Four of these automatic scales have been in use during the past four summers at Akron, Colo., and continuous records have been secured during these periods. The results of these measurements will be discussed in other papers.

A brief review is also given of other forms of transpiration balances, which are divided into two classes: Those operating on the step-by-step principle, which includes the balances here described, and those of the continuous-record type. The first class includes balances in which the adjustment is secured by adding small weights (solid or liquid) of equal mass or by moving a counterpoise in uniform steps. In the second class the plant is suspended from a spring, or from a variable lever, or is mounted (directly or indirectly) on a float.

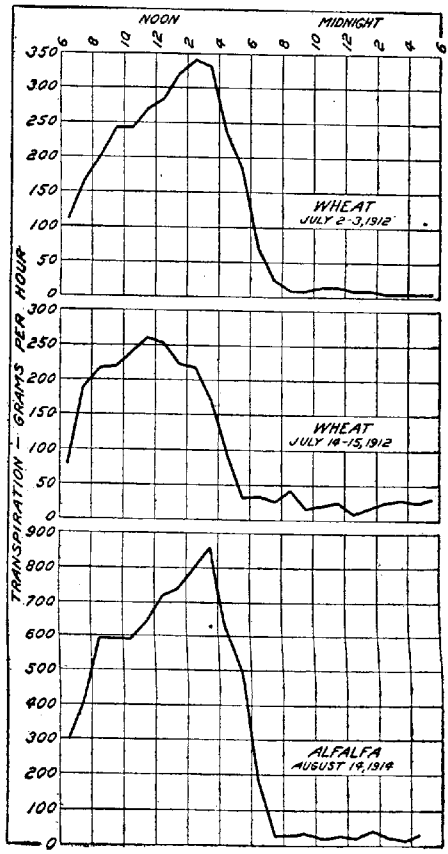

FIG. 18.—Transpiration graphs corresponding to the three records given in figure 16, plotted in rectangular coordinates.

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PLATE IX

Fig. 1.—Four automatic balances in operation at Akron, Colo., June 19, 1912, with the front of the box containing the mechanism open. The recording device is shown just beyond the first box. A separate recorder is used for each instrument.

Fig. 2.—Automatic balances, Akron, Colo., July 24, 1912; boxes closed and recorders covered. Except when being adjusted, this is the condition in which the apparatus is maintained.


1


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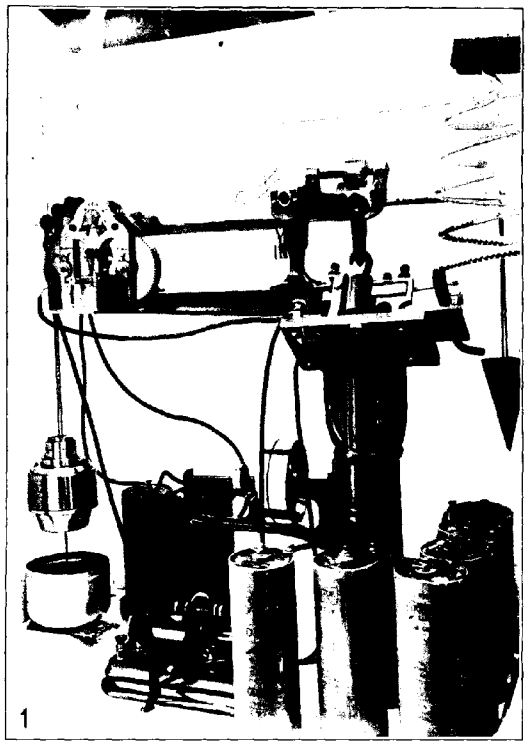
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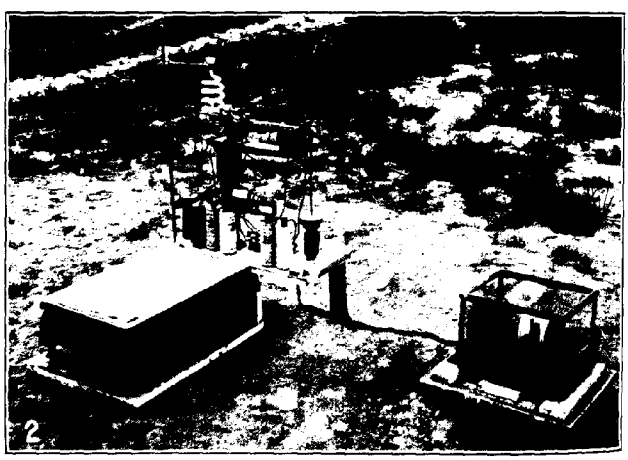
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PLATE X

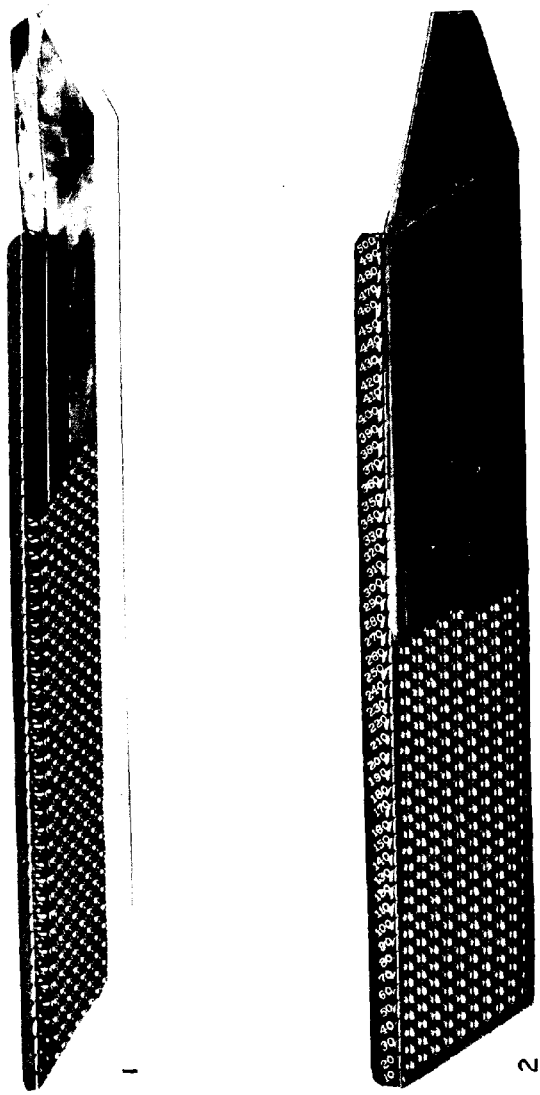
Fig. 1.—Front of balance, cover removed, showing mechanism. The spiral glass ball container will be seen in the upper right-hand corner, the balls passing down through the ball dropper into the basket shown at the extreme right. The spring motor for raising the beam is shown at the upper left-hand side. The dashpot is seen below the weight carrier.

Fig. 2.—General view of automatic balance with case removed.

PLATE XI

Fig. 1.—Measuring tray used in counting total number of balls delivered to the container on the balance arm during the 24-hour period.

Fig. 2.—Another view of the measuring tray looking vertically downward on the tray, showing the 60° angle which the base makes with the graduated side. This tray contains 255 balls, as will be seen by reference to the graduations.



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PARASITISM OF COMANDRA UMBELLATA

By GEORGE GRANT HEDGCOCK,
Pathologist, Investigations in Forest Pathology,
Bureau of Plant Industry

One of the most important and most injurious of the stem or blister rusts occurring on pines is *Peridermium pyriforme* Peck, which attacks *Pinus (murrayana) contorta* Loud., *P. ponderosa* Laws., and *P. ponderosa scopulorum* Engelm. in the western United States, *P. divaricata* Du Mont de Cours. in the Northern States, and *P. pungens* Michx. and *P. rigida* Mill. in the Northwestern States. *Peridermium pyriforme* is a heterœcious rust and is dependent for its existence upon its alternate, or summer, stage, which occurs on species of Comandra.

The problem of the eradication of this important rust being so intimately associated with plants of *Comandra* spp. led the writer to investigate their manner of growth and means of propagation. It was found that the plants of at least two species, *C. pallida* A. DC. and *C. umbellata* (L.) Nutt., have apparently become largely dependent on parasitism for their continued existence. The other two North American species, *C. livida* Richards, and *C. richardsiana* Fernald, resemble the former species in appearance and habit and are probably equally parasitic in their nature.

The writer has carefully examined the root system of living plants of both *C. umbellata* and *C. pallida*, but only of dried specimens of the other two species. The former have long underground rootstocks which bear here and there small roots or rootlets usually less than 5 inches in length. These rootlets branch sparsely and are nearly always attached to the roots or underground stems of other species of plants. At the point of attachment there is formed by the root of *Comandra* spp. a nearly hemispherical disk or holdfast. This holdfast is either superficial or slightly embedded in the cambium layer of tissues of the host, but does not send out haustoria, as is the case in species of Razoumofskya on the limbs and trunks of coniferous trees. The chief function of the roots of *Comandra* spp. appears to be that of attachment to host plants for the purpose of obtaining nourishment and a water supply. Plants of *Comandra* spp. frequent dry, rocky soils, which often have a low water content.

Plants of all these species of Comandra bear leaves; and although attached as parasites to the roots of other plants, they are not entirely dependent upon their host plants for organic compounds, since they are able to further elaborate these compounds in the liquids received from

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their hosts. In this respect their development is similar to that of plants of species of Phoradendron.

Both *C. umbellata* and *C. pallida* very commonly are associated with and parasitic upon species of Vaccinium, but are not at all dependent upon this genus for host plants. This has especially been noted in the case of *C. pallida* in the States of Colorado, Montana, Nebraska, South Dakota, and Wyoming, and in *C. umbellata* in the States of Connecticut, Maryland, Michigan, Minnesota, New Jersey, New York, Pennsylvania, Vermont, Virginia, and Wisconsin, and the District of Columbia. Plants of both species are parasitic upon a great variety of plants belonging to widely different sections of the Spermatophyta. No attachment to plants of any member of the Pteridophyta has been noted.

C. umbellata has been found by the writer as a parasite on the roots of the following species of plants in the Eastern States:

Acer rubrum L.
Achillea millefolium L.
Andropogon virginicus L.
Angelica villosa (Walt.) B. S. P.
Antennaria plantaginifolia (L.) Richards.
Aster ericoides L.
Aster macrophyllus L.
Aster patens Ait.
Aster undulatus L.
Baptisia tinctoria (L.) Br.
Betula nigra L.
Betula populifolia Marsh.
Carex sp.
Castanea dentata (Marsh.) Borkh.
Chimaphila umbellata (L.) Nutt.
Chrysopsis mariana (L.) Nutt.
Comptonia peregrina (L.) Coulter.
Danthonia compressa Austin.
Fragaria americana (Porter) Britton.
Fragaria virginiana Duchesne.
Gaylussacia frondosa (L.) T. and G.
Hieracium venosum L.
Ionactis linariifolius (L.) Greene.
Lespedeza violacea (L.) Pers.
Lysimachia quadrifolia L.
Meibomia paniculata (L.) Kuntze.
Panicum sp.
Poa compressa L.
Poa pratensis L.
Populus tremuloides Michx.
Potentilla monspeliensis L.
Quercus coccinea Muenchh.
Quercus digitata (Marsh.) Sudw.
Quercus marilandica Muenchh.
Quercus nana (Wood) Britton.
Rhus copallina L.
Rosa blanda Ait.
Rosa canina L.
Rubus canadensis L.
Rubus procumbens Muhl.
Rubus villosus Ait.
Solidago bicolor L.
Solidago caesia L.
Solidago juncea Ait.
Solidago nemoralis Ait.
Solidago speciosa Nutt.
Spiraea salicifolia L.
Vaccinium atrococcum (A. Gray) Heller.
Vaccinium nigrum (Wood) Britton.
Vaccinium vacillans Kahn.

In addition to the foregoing and incomplete list there must be added at least three unidentified species of grasses.

During the last three years a number of attempts, with varying success, have been made at Washington, D. C., to grow plants of *C. umbellata* and *C. pallida*, both by germinating the seed and by transplanting rootstocks to beds and pots in greenhouses. In every case where living rootstocks unattached to host plants have been transplanted to pots or

beds without the host plants present, little or no growth on the part of the plants of *Comandra* spp. has taken place, and the plants eventually died. Successful results in growing these species have been accomplished by only two methods: First, by transplanting sods containing the plants of *Comandra* spp. from out of doors to the greenhouse without breaking the attachments of the roots of the parasite to those of the host; second, by planting seed in flats in the fall out of doors and germinating them in the presence of the roots of host plants after exposing the seeds to freezing temperatures by allowing the flats to remain out of doors all winter.

Dr. E. P. Meinecke, of the Office of Forest Pathology, reports by letter that he has three plants of *C. umbellata* raised from seed sown in 1913, which remained dormant till 1915, when they germinated and grew without any host plant. These plants were 5 inches high on July 17, 1915. This is positive proof that this species of Comandra can live without parasitism if necessary. It remains to be seen whether these plants will continue to grow indefinitely without the presence of host plants.

The results from our experiments indicate that when the rootstocks of plants of *Comandra* spp. are broken entirely loose from their root attachment to host plants they usually die through an inability to reattach themselves. These new data on a subject which apparently has not been previously investigated indicate a greater degree of parasitism in species of Comandra than has hitherto been suspected, and will render more obvious the desirability of the destruction of plants of *Comandra* spp. in the vicinity of forest-tree nurseries.

SEPARATION OF SOIL PROTOZOA[1]

By NICHOLAS KOPELOFF, H. CLAY LINT, and DAVID A. COLEMAN,
Research Fellows, The New Jersey College for the Benefit of Agriculture and Mechanic Arts

Some interesting problems have been suggested by the contention of Russell and Hutchinson (9, 10)[2] that protozoa are one of the limiting factors in soil fertility, because they feed upon and consequently limit the numbers of soil bacteria. Before the agricultural scientist can successfully formulate a complete explanation of the phenomena concerned with the function of protozoa in soils it is essential to establish certain fundamentals in methodology. Russell and Hutchinson (9, 10) and Cunningham (2, 3) have presented some valuable information concerning the depression of bacterial numbers as a result of inoculation with cultures of protozoa. The writers entered upon an investigation of a similar nature, with an attempt to base their work upon the use of protozoa-free cultures of bacteria, and bacteria-free cultures of protozoa.

But little mention is to be found in the literature regarding the separation of the different kinds of protozoa from each other and from bacteria. Russell and Hutchinson (9, 10) and Fred (4) have employed an efficient method of filtration for obtaining cultures of protozoa, but they do not offer any further experimental data concerning such separations. Cunningham (2, 3) has made use of a single-drop method for obtaining protozoa-free cultures of bacteria, based on the transfer to a suitable medium of a drop from a protozoan culture which upon microscopic examination revealed no protozoa. On the other hand, he does not describe any direct method for obtaining a bacteria-free culture of protozoa. Jordan (5, p. 469) mentions a method which might prove somewhat tedious—that is, having protozoa pass through concentric rings of dead bacteria on a culture plate until they had no living adhering bacteria. He refers also to Frosch's[3] method of separation by means of a sodium-carbonate solution. Richter (8) suggests the use of a high-gelatin medium which would suppress the bacterial growth of liquefying organisms. Biffi and Razzeto (1) give an account of the passage of protozoa through semipermeable filters after a considerable period of time has elapsed.

The writers are in agreement with Biffi and Razzeto regarding the importance of the time element in filtration, since it has been observed that protozoa have been able to work through the pores of a filter in a short time.

In the work under consideration—namely, the separation of flagellates from ciliates—an 8-day-old culture of soil organisms was employed.

[1] From the Departments of Soil Chemistry and Bacteriology, New Jersey Experiment Station, New Brunswick, N. J.

[2] Reference is made by number to "Literature cited," p. 139-140.

[3] Frosch, P. Zur Frage der Reinzüchtung der Amöben. *In* Centbl. Bakt. [etc.], Abt. 1, Bd. 21, No. 24/25, p. 926-932. 1897.

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This was prepared by adding 100 gm. of Penn clay loam soil to 1 liter of a 10 per cent hay infusion plus 0.5 per cent of egg albumin, which the writers had previously found to be best adapted to the large and rapid development of protozoa in such soil (6).

The method of procedure was as follows: The numbers of protozoa in the stock culture solution were first counted by the new method described in a previous paper (6) and recorded under classes of (1) flagellates, (2) small ciliates (12 to 20μ), and (3) large ciliates (25 to 60μ). No amœbæ developed in the short period of incubation. Ten c. c. of the culture solution were then placed (by means of a sterile pipette) on filter paper, previously sterilized with alcohol, and allowed to filter through for one minute. The protozoan content of the filtrate was then recorded in triplicate and the filtrate incubated for five days at 22° C., in order to allow the excystment of any encysted forms. The filtration and incubation processes were then repeated, if necessary, until all the living protozoa of the desired type had been separated out. The filter paper was used in from one to five different thicknesses (Schleicher and Schüll's No. 589). The results are recorded in Table I.

TABLE I.—*Number of protozoa per 10 c. c. of filtrate through varying thicknesses of filter paper*

Number of filter papers.	Sample No.	Number of flagellates.	Number of small ciliates, 12–20μ.	Number of large ciliates, 25–60μ.	Total.
0[a]	1	106, 250	53, 125	42, 500	201, 875
	2	127, 500	42, 500	31, 875	201, 875
	3	85, 000	21, 250	81, 875	188, 125
Average		106, 250	38, 958	52, 083	197, 292
1	1	63, 750	53, 125	0	116, 875
	2	63, 750	31, 875	0	95, 625
	3	74, 375	31, 875	0	106, 250
Average		67, 293	38, 958	0	106, 246
2	1	53, 125	31, 875	0	85, 000
	2	53, 125	21, 250	0	74, 375
	3	73, 750	21, 250	0	95, 250
Average		60, 416	24, 742	0	85, 208
3	1	53, 125	10, 625	0	63, 750
	2	53, 125	10, 625	0	63, 750
	3	63, 750	10, 625	0	73, 750
Average		56, 666	10, 625	0	67, 083
4	1	10, 625	0	0	10, 625
	2	10, 625	0	0	10, 625
	3	10, 625	0	0	10, 625
Average		10, 625	0	0	10, 625
5	1	None.	None.	None.	None.
	2				
	3				
Average					

[a] Stock protozoan solution.

It will be observed from Table I that the large ciliates are not able to pass through the filter paper at all, which fact is in agreement with the experience of Russell and Hutchinson (9, 10). The noteworthy feature, however, is that the number of small ciliates decreases rapidly in increasing the thicknesses of the filter paper from two to four. Thus, with four thicknesses of filter paper all of the ciliates found in the solution employed were separated from the flagellates. Likewise it was a simple matter to separate the small from the large ciliates. In this way it becomes possible to employ mass cultures of flagellates, small ciliates, or large ciliates, as may be necessary in the problems indicated at the outset.

In an effort to determine the effect of filtration on the separation of soil protozoa from bacteria, a bacterial count was made of the stock-culture solution previously employed, known to contain soil microorganisms. Ten c. c. of this solution were then filtered through five thicknesses of sterilized (with alcohol) filter paper (S. & S. No. 589). The residue on the filter paper, consisting of all of the protozoa originally present, together with some adhering bacteria, was then plated out on Lipman and Brown's (7, p. 132) synthetic agar. The bacterial count showed that 90 per cent of the bacteria had passed through the filter paper (after making due deduction for contamination from the air by exposing agar plates for the same length of time as was necessary for filtration), thus leaving the protozoan residue comparatively free from bacteria.

This method in all probability would not allow complete separation of the protozoa from the bacteria. Consequently the work was not carried out any farther. However, this method, because of its rapidity and simplicity, might prove of value in investigations concerned with the effect of protozoa on mixed but not on pure cultures of bacteria.

While these preliminary experiments do not warrant any definite conclusions, they are, nevertheless, indicative of some of the difficulties which the soil protozoologist encounters.

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